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Protein phosphatase 1 regulates phosphorylation of Gasdermin D and pyroptosis

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Experimental

Materials and Methods

Reagents

Reagents	Source	Identifier
PPP1CA shRNA(F1)	Sigma	--
PPP1CA shRNA(F2)	Sigma	--
PPP1CB shRNA(F7)	Sigma	--
PPP1CB shRNA(F9)	Sigma	--
CHX	Millipore	5.08739
MG132	Sigma	M8699
OA	Shanghai yuanye Bio-Technology	S30686
DAPI	Beyotime	C1006
LPS	Sigma	82857-67-8
Nigericin	MedChemExpress	HY-127019
Anti-Flag M2 Magnetic Beads	Sigma	M8823
TRIzol Reagent	Invitrogen	15596018
DiI	Beyotime	C1036
PhosTag	Wako Chemicals	304-93521
Chloroquine phosphate (CQ)	Sigma	PHR1258
PMA	Sigma	P1585
Protease Inhibitor Cocktail	Thermo Fisher Scientific	78430
Phosphatase inhibitor	Thermo Fisher Scientific	A32957
Antibodies	**Source**	**Identifier**
Anti-Flag tag pAb	MBL	PM020
Anti-β-Actin mAb	Cell Signaling Tech	3700S
Anti-GSDMD mAb	Abcam	Ab210070
Anti-cleaved N-terminal GSDMD mAb	Abcam	ab215203
Alexa Fluor 488 goat anti-Rabbit IgG(H+L)	Invitrogen	A11034
Alexa Fluor 568 goat anti-Mouse IgG(H+L)	Invitrogen	A11031
Plasmids	**Source**	**Identifier**
pCMV-3Tag (Flag)	Agilent Technologies	240195
pCMV-3Tag (Myc)	Agilent Technologies	240196
pLKO.1	Addgene	10878
pMD2.G	Addgene	12259
psPAX2	Addgene	12260
pECMV-PPP1CA-Myc	MiaoLing bio	P10025
pECMV-PPP1CC-Myc	MiaoLing bio	P10026

Critical Kits	Source	Identifier
iScript cDNA synthesis Kit	Bio-Rad	1706691
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Fast Mutagenesis System	Transgen	FM111
iTaq Universal SYBR Green Supermix	Bio-Rad	172521
pEASY-Uni Seamless Cloning and Assembly Kit	Transgen	CU101
SuperSignal™ West Pico PLUS Substrate Kit	Thermo Fisher Scientific	34578
LDH Cytotoxicity Assay Kit	Beyotime	C0017
Human TNF ELISA set	BD	555212
Lipofectamine™3000 Transfection Reagent	Thermo Fisher Scientific	L3000001
Human IL-1β ELISA Set II	BD	557953

Cell lines and treatments.

THP-1 and HEK293T cells were obtained from American Type Culture Collection (ATCC, Maryland, USA). HeLa GSDMD KO cells were provided by Dr. Feng Shao. HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose containing media at 37℃ with 5% CO_2. THP-1 cells were cultured in myeloid medium comprised of RPMI 1640 medium. The culture medium was supplemented with 1X Penicillin-Streptomycin and 10% (v/v) fetal bovine serum (FBS).

Immunoblot analysis.

Cell extracts were prepared using NP-40 lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% SDS, 0.5% deoxycholate) supplemented with a complete protease inhibitor cocktail (Roche) and a PhosSTOP phosphatase inhibitor cocktail (Roche). Samples were subjected to SDS–PAGE and the resolved proteins were then transferred to a polyvinylidene fluoride membrane (Millipore). Immunoblots were probed with indicated antibodies and visualized using a SuperSignal West Pico chemiluminescence ECL kit (Pierce).

Coimmunoprecipitation.

HEK293T cells were transfected with 3×flag-GSDMD and the indicated PP1 expression plasmids for 24 h. Then cells were lysed by NP-40 lysis buffer. After centrifugation, supernatants were co-incubated with Anti-Flag M2 magnetic beads for overnight. The beads were separated by magnetism followed by immunoblot analysis with indicated antibody.

Mass spectrometry and sample preparation.

HeLa GSDMD KO cells were transfected with 3×flag-GSDMD plasmids for 24 h. After isolation by Anti-Flag M2 magnetic beads, proteins were run on an SDS-PAGE gel and stained with Coomassie brilliant blue in 50% methanol and 5% acetic acid. The indicated Gel bands were cut into 1mm size pieces and placed into separate 1.5ml polypropylene tubes. Then 100µl of 50% acetonitrile in 50mM ammonium bicarbonate buffer was added to tube and the samples were then incubated at room temperature for 20min. Then, the gel slice was incubated with 55mM iodoacetamide (in 50mM ammonium bicarbonate) for 45min in the dark at room temperature, before the gel was washed sequentially with 50mM ammonium bicarbonate, water and acetonitrile. Samples were then dried in a Speedvac for 20min. Trypsin (Promega Corp.) (10ng/µl in 25mM ammonium bicarbonate, pH8.0) was added to each sample tube to just cover the gel, and samples were then incubated at 37 °C for overnight.

The peptides were separated by an 85 min gradient elution at a flow rate 0.30 µl/min with a Thermo-Dionex Ultimate 3000 HPLC system, which was directly interfaced with a Thermo Scientific Q Exactive mass spectrometer. The analytical column was a home-made fused silica capillary column (75 µm ID, 150 mm length; Upchurch, Oak Harbor, WA) packed with C-18 resin (300 Å, 5 µm, Varian, Lexington, MA). Mobile phase consisted of 0.1% formic acid, and mobile phase B consisted of 80% acetonitrile and 0.1% formic acid. The Q Exactive mass spectrometer was operated in the data-dependent acquisition mode using Xcalibur 2.2 software and there was a single full-scan mass spectrum in the orbitrap (300-1800 m/z, 70,000 resolution) followed by 20 data-dependent MS/MS scans at 27% normalized collision energy (HCD).

The MS/MS spectra from each LC-MS/MS run were searched against the

GSDMD.fasta from UniProt using an in-house Proteome Discoverer (Version PD1.4, Thermo-Fisher Scientific, USA). The search criteria were as follows: full tryptic specificity was required; two missed cleavage was allowed; Carbamidomethyl (C) were set as the fixed modifications; the oxidation (M) and phosphorylation (STY) were set as the variable modification; precursor ion mass tolerances were set at 20 ppm for all MS acquired in an orbitrap mass analyzer; and the fragment ion mass tolerance was set at 0.02Da for all MS2 spectra acquired. The peptide false discovery rate (FDR) was calculated using Percolator provided by PD. When the q value was smaller than 1%, the peptide spectrum match (PSM) was considered to be correct. FDR was determined based on PSMs when searched against the reverse, decoy database. Peptides only assigned to a given protein group were considered as unique. The false discovery rate (FDR) was also set to 0.01 for protein identifications.

Immunostaining and confocal microscopy.

After transfecting with indicated plasmids for 24h, cells grown on coverslips were fixed for 15 min with 4% paraformaldehyde in PBS, permeabilized for 5min in 0.4% Triton X-100 in PBS and blocked using 3% BSA for 1h. Then, cells were stained with the indicated primary antibodies followed by incubation with fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole) (Sigma-Aldrich). Slides were mounted using Aqua-Poly/Mount (Dako). Images were captured using a laser scanning confocal microscope (Olympus Fluoview FV1000 Confocal System) with a ×63 water immersion objective and Olympus Fluoview software (Olympus). All confocal images are representative of three independent experiments.

LDH release assay.

HEK-293T cells were transfected with the indicated GSDMD. Four hours after transfection, medium was changed. Forty-eight hours after the transfection, LDH release was measured. For cell death in THP-1, cells were plated at 50,000 cells per well in a 96-well plate. Cells were stimulated with ultrapure 200 ng/ml LPS (and

okadaic acid with indicated concentration if applicable) for 3 hours before stimulation with nigericin. One hour after stimulation, LDH release was measured according to the manufacturer's instructions.

Measurement of cytokines.

Cells were stimulated with ultrapure LPS (200 ng/ml) (InvivoGen) for 3 hours before stimulation with nigericin. One hour after stimulation, concentrations of IL-1β in culture supernatants were measured by ELISA kit (R&D Systems) according to the manufacturer's instructions.

Measurement of phosphorylated proteins.

Supplements of 50 μM phos-tag and 10 mM $MnCl_2$ were added during preparation of SDS-PAGE gel. And after running SDS-PAGE gel, the gel was washed three times with an added 10 mM EDTA running buffer. Others were same as above protocol of immunoblot analysis.

Recombinant Protein Expression and Purification.

For recombinant expression of the p20/p10 complex of catalytically active caspase-4 in *E. coli*, the previous reported methods were used. Simply, cDNAs encoding the p20 and p10 fragments were cloned into a single pACYCDuet vector with the p20 subunit fused with an N-terminal 6 × His tag. The plasmid was transformed into *E. coli* BL21 (DE3). The bacteria were cultured in LB medium with appropriate antibiotics. Protein expression was induced overnight at 20˚C with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) after OD_{600} reached 0.8. Cells were lysed in buffer A containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, and 10 mM β-mercaptoethanol. The p20/p10 complex was purified by Ni-Sepharose beads (GE Healthcare Life Sciences) in buffer A, and eluted with buffer B containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 300 mM imidazole, and 10 mM β-mercaptoethanol. The eluted proteins were further purified by HiTrap Q anion exchange and Superdex 200 Increase size exclusion chromatography. To express wild-type GSDMD_full and its

mutants, the relative cDNAs were cloned into the pGEX-6P-1 vector with an N-terminal GST tag. The plasmid was transformed into *E. coli* BL21 to express proteins. Cells were lysed in buffer C containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl. The GST-tag GSDMD were purified by Glutathione Sepharose in buffer A. Then, GST tag was cleaved by HRV 3C protease overnight at 4˚C. All purified proteins were stored at -80˚C in buffer D containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 5 mM DTT.

Protein–lipid binding assay.

Cardiolipin (0.5 mg/ml, 2ul) was stained on PVDF membrane after activation in methanol. To block non-specific binding, the lipid membranes were preincubated with binding assay buffer (3% fatty acid-free BSA (Sigma) in PBS) for 1 h at room temperature. The indicated GSDMD_full (2 mg/ml) were incubated with active caspase4 (0.5 uM) in buffer (20 mM HEPES (pH 7.5), 150 mM NaCl) for 1 h at room temperature to product GSDMD-NT, respectively. Then, lipid membranes were incubated with the GSDMD-caspase4 mixture (diluted to 2 ug/ml GSDMD) diluted in binding assay buffer for 1 h at room temperature and then washed three times (5 min each time) with wash buffer (0.1% Tween-20 in PBS, PBST). Membrane-bound proteins were detected by GSDMD NT primary antibodies diluted in binding assay buffer for 1 h at room temperature, followed by incubation for 1 h with horseradish-peroxidase-conjugated secondary antibody diluted 1:2000 in binding assay buffer. After washing three times with wash buffer, proteins were visualized using a SuperSignal West Pico chemiluminescence ECL kit (Pierce).

Liposome preparation.

Phosphatidylcholine (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 10 mg/ml in chloroform; 20 μl), PE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 10 mg/ml in chloroform; 20 μl) and cardiolipin (CL, 1’,3’-bis(1,2-dioleoyl-sn-glycero-3-phospho)-sn-glycerol (sodium salt), 25 mg/ml in chloroform; 20 μl) were mixed and

the solvent was evaporated under a stream of N_2 gas. The lipid mixture was suspended in 900 ul buffer (20 mM HEPES (pH 7.4), 150mM NaCl) for 10 min. The suspension was pushed through 100 nm Whatman Nuclepore Track-Etched Membrane 21 times to obtain homogeneous liposomes (1.5 mM lipids).

Liposome binding assay.

Liposomes were prepared as abovementioned method. For liposome binding assay, the indicated GSDMD (5 uM) treated by caspase 4 were incubated with liposomes in 180 μl buffer (20 mM HEPES (pH 7.4), 150mM NaCl) for 30 min at room temperature before sedimentation at 140,000 g for 20 min at 4 °C. Supernatants were removed immediately and the pellets were washed twice with buffer (20 mM HEPES (pH 7.4), 150mM NaCl) and then resuspended in an equal volume of buffer. Proteins in both pellets and supernatant were then analysed by SDS PAGE and immunoblot.

Formation of GSDMD oligomer on liposomes.

The indicated GSDMD_full (10 uM) were cleaved by caspase4 as the abovementioned method. The GSDMD-caspase4 mixtures (20 ul) were incubated with containing cardiolipin liposome (180 ul, 300 uM lipids), or buffer (20 mM HEPES (pH7.4, 180 ul) for 1 h to form oligomer. Then, the mixtures were treated 3 times dilution by NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.5% (w/v) IgePal, 50 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail), and further diluted by native protein loading buffer (no β-mercaptoethanol and SDS) for native gel electrophoresis analysis. Samples were resolved by native gel electrophoresis and transferred to PVDF membranes and analyzed by immunoblot. Immunoreactivity was visualized by a SuperSignal West Pico chemiluminescence ECL kit (Pierce).

Figure S1

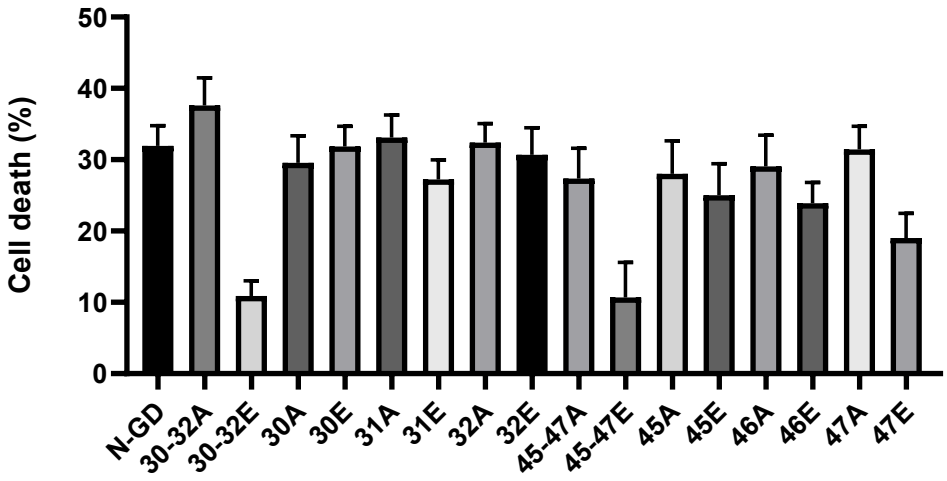

Fig. S1 Cytotoxicity activity of the indicated GSDMD-NT constructs as measured by lactate dehydrogenase (LDH) release in 293T cells transfected with the indicated GSDMD for 48 h.

Figure S2

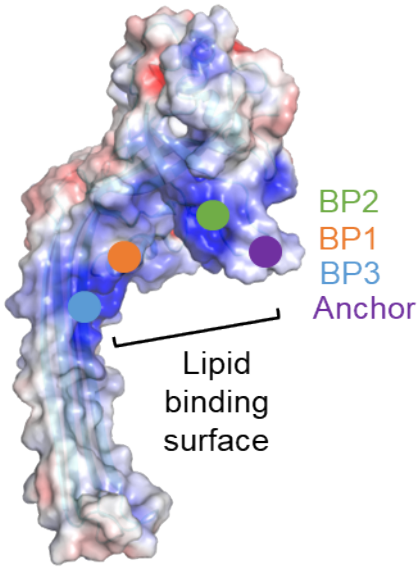

Fig. S2 Location of lipid binding region containing the three BPs and an anchor in GSDMD-NT from GSDMD pore structure (PDB: 6VFE).

Figure S3

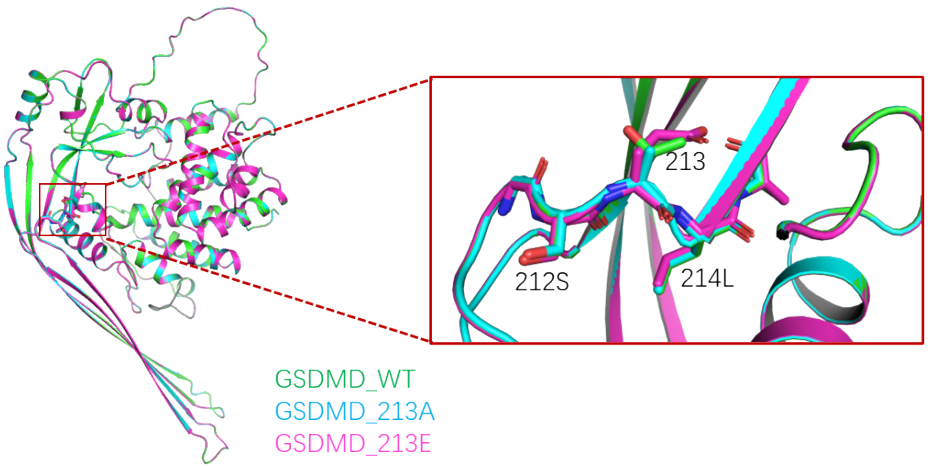

Fig. S3. The 3D structures of GSDMD_NT and its mutants after energy minimization.

Figure S4

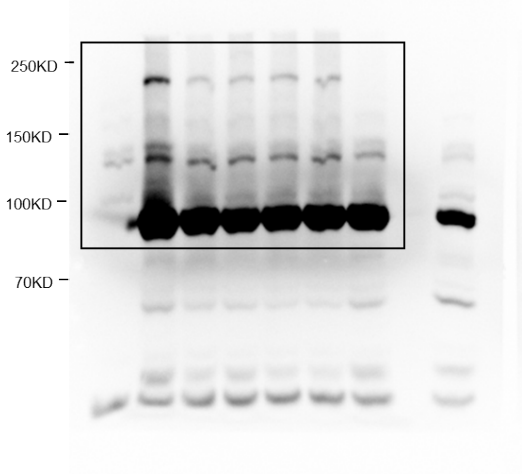

Fig. S4. The raw image for Fig. 1 in the manuscript. The highlighted part in black frame was showed in the manuscript.

Figure S5

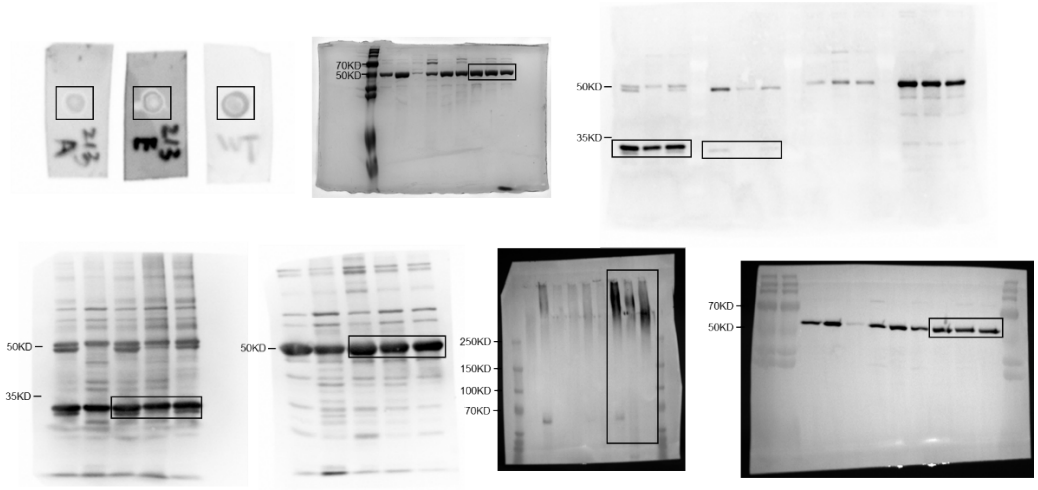

Fig. S5. The raw images for Fig. 2 in the manuscript. The highlighted parts in black box were showed in the manuscript.

Figure S6

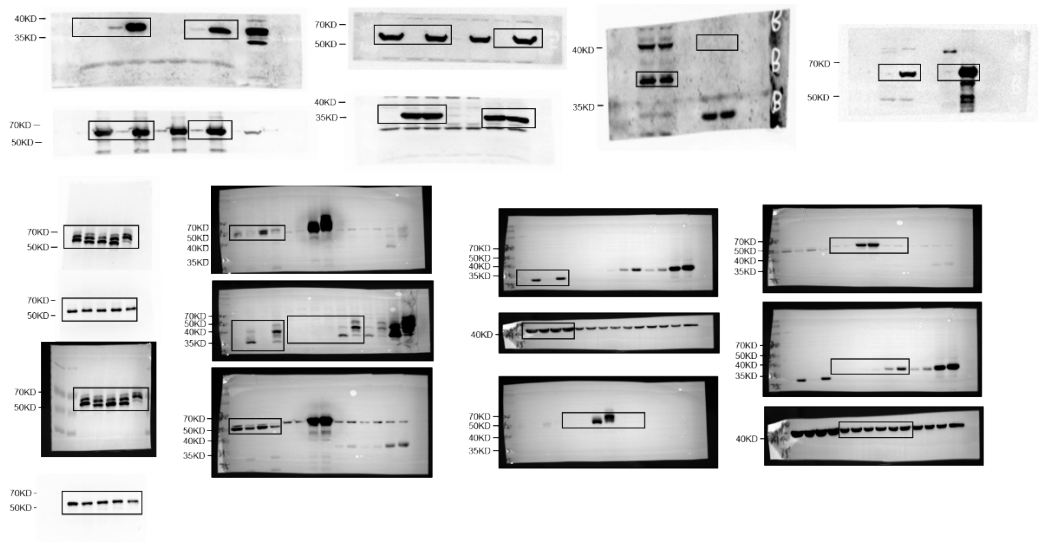

Fig. S6. The raw images for Fig. 3 in the manuscript. The highlighted parts in black box were showed in the manuscript

Table S1

Table S1. The energy and ΔΔG value after energy minimization.[a]

Protein	Potential Energy-OPLS 2005		Potential Energy-OPLS3	
GSDMD_WT	-1500.958		-1835.018	
GSDMD_T213A	-1496.097	ΔΔG = +4.861	-1838.491	ΔΔG = -3.473
GSDMD_T213E	-1497.32	ΔΔG = +3.638	-1836.854	ΔΔG = -1.836

[a] The energy minimization was performed using OPLS 2005 and OPLS3 methods in Maestro.